AD_____

Award Number: W81XWH-10-2-0116

TITLE: “Treating Combat Hearing Loss with Atoh1 Gene Therapy”

PRINCIPAL INVESTIGATOR: Andrew K. Groves Ph.D.

CONTRACTING ORGANIZATION: Baylor College of Medicine, Houston, TX 77030

REPORT DATE: October 2012

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED *(From - To)*
October 2012	ANNUAL	1 Oct 2011 - 30 Sep 2012

4. TITLE AND SUBTITLE
"Treating Combat Hearing Loss with Atoh1 Gene Therapy"

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-10-2-0116

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Andrew K. Groves Ph.D.

email: akgroves@bcm.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Baylor College of Medicine,
Houston, TX 77030

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The goal of the project is to test experimental therapies for hearing loss in transgenic mouse models. We have developed molecular methods to identify sensory hair cells in the inner ear, including two different validated genetic profiles of sensory hair cells. We have generated and derived genetically modified mice to serve as models for treating hearing loss with gene therapy. We have shown that five such models are not suitable for our studies, but we have genetically engineered two new mouse lines line and re-derived a third line from our collaborators. We have also generated data to determine the genetic differences in supporting cells of the cochlea at two different ages, and to determine the response of these cells to pharmacological inhibitors that may promote hair cell regeneration.

15. SUBJECT TERMS
Noise-induced hearing loss, Mouse models, Gene Therapy, Regeneration

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	14	19b. TELEPHONE NUMBER *(include area code)*

TABLE OF CONTENTS

INTRODUCTION:

Hearing and balance dysfunction are frequently seen in military personnel exposed to blast injuries. The most common cause of these disorders is the death of inner ear sensory hair cells. Once hair cells are killed, they are not replaced and combat-induced hearing and balance disorders are therefore permanent. At present, there is no treatment that allows the replacement of these sensory cells. The long term goal of this research is to develop gene or drug-based therapies that allow the regeneration of sensory hair cells and the restoration of hearing and balance in combat personnel. The transcription factor **Atoh1** is one of the first genes to be switched on when hair cells form. Atoh1 has been shown to induce new hair cells when activated in embryonic or neonatal inner ears. We will attempt to re-activate Atoh1 by genetic or pharmacological methods in an animal model to test its ability to promote regeneration of sensory hair cells.

BODY OF REPORT:

Aim 1: To determine the genetic targets of Atoh1

We proposed the following deliverables for Year 2:

1. We will produce a validated list of Atoh1 targets from our combined microarray and ChIP-SEQ experiments. *As described in the Research Plan, the ChIP-SEQ experiments are technically difficult and may require up to 24 more months to complete. We can still gain insights into Atoh1 targets in the absence of this data.*

Results:

1. As discussed in the previous reports for this year, we have enhanced our list of genes expressed in hair cells by using RNA-sequencing to analyze hair cell-specific transcripts from purified hair cells:

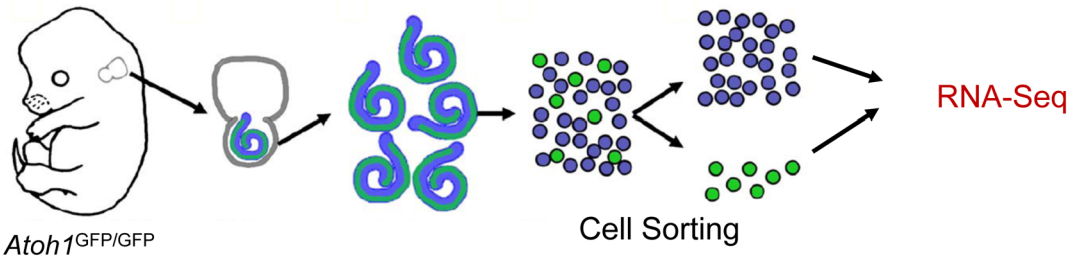

Figure 1: Fluorescence activated cell sorting allows us to purify Atoh1-GFP cells from dissociated neonatal mouse cochleas. RNA from the GFP-positive and negative populations are purified and analyzed by RNA-sequencing.

The data from replicate samples was highly reproducible (r=0.98). Below we show the top ten genes enriched in hair cells by this analysis. Three of the genes contain a candidate binding site for the Atoh1 transcription factor, suggesting they may be direct targets of Atoh1

ProbeID	Symbol	FoldChange	P-value	Presence of AtEAM Motif
NM_011887	Scn11a	797.8707417	8.50E-140	AACAGATGGC:-4538;
NM_029182	Rasd2	507.2342205	6.45E-136	
NM_026571	Lhfpl5	573.7689179	5.35E-135	
NM_001195091	Gm6537	492.1156572	4.09E-134	
NM_001099331	R3hdml	604.6938191	2.34E-132	
NM_177822	Mslnl	870.3430246	2.05E-131	AGCAGCTGTC:-3796;
NM_007500	Atoh1	474.2575833	6.23E-130	TACAGATGGC:770;
NM_001033292	Espnl	432.6407015	1.52E-129	
NM_148413	Myo3a	618.7313932	1.51E-128	
NM_001039653	Lhx3	452.0873136	5.58E-128	

2. Last year, we attempted ChIP-SEQ experiments to identify Atoh1 targets using whole cochleas from newborn mice. The advantage of this method was that it yielded a substantial amount of tissue. However, since hair cells represent less than 1% of the total cells in a newborn cochlea, the target cells are at a very low purity, and this likely accounted for the unacceptably high signal-to-noise ratio we obtained in the first round of ChIP-seq. This year, we modified our approach by generating mice which carried two alleles of Atoh1 tagged with a FLAG epitope, together with a transgene expressing GFP under control of an Atoh1 enhancer. Generating sufficient mice with correct genotype took longer than originally planned due to issues with breeding, however This allowed us to first purify hair cells, and then perform ChIP-seq on the purified hair cells using antibodies to the FLAG epitope:

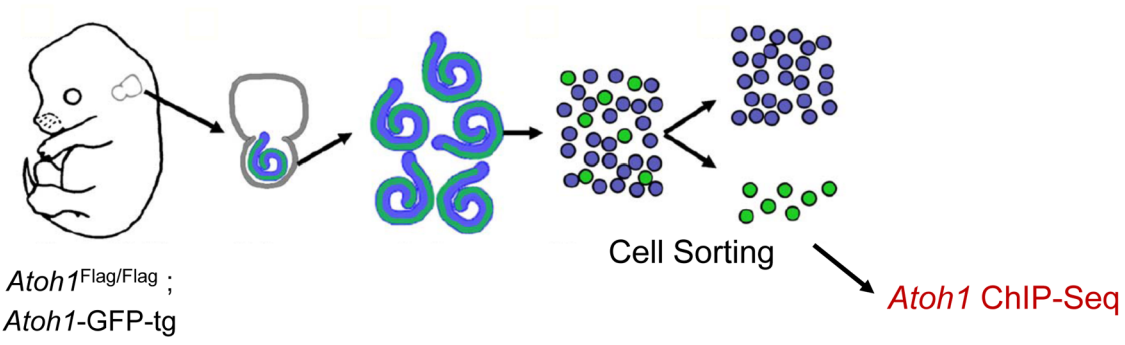

Figure 2: Fluorescence activated cell sorting allows us to purify Atoh1-GFP cells from dissociated cochleas in which the hair cells express two alleles of a FLAG-tagged version ofAtoh1. DNA containing the bound tagged Atoh1 protein can then be immunoprecipitated and analyzed by sequencing.

We purified 250,000 hair cells from 125 newborn mice. Unfortunately, the amount of immunoprecipitated DNA was not enough to give a robust signal, making sequencing impossible. We therefore used an alternative approach to obtain Atoh1 targets, using cross-referencing from Atoh1 ChIP-seq from the cerebellum performed by our colleagues at Baylor. By comparing our hair cell RNA-seq list with the cerebellum ChIP-seq list, we identified 23 genes whose enhancers are know to be bound by Atoh1 that are also enriched in hair cells:

Accession	Gene	Presence of AtEAM Sites?
NM_029440	4930434E21Rik	GCCATCTGGA:-427;
NM_007500	Atoh1	TACAGATGGC:770;
NM_019446	Barhl1	
NM_029426	Brsk2	CCCATCTGCC:839;
NM_028055	Btbd17	
NM_007583	Cacng2	
NM_015795	Fbxo16	GCCATCTGCT:-495;
NM_019479	Hes6	
NM_018741	Igfbpl1	
NM_008595	Mfng	GCCATCTGTG:-1466;
NM_001113199	Naca	GCCACCTGTT:-2207;
NM_009717	Neurod6	
NM_027032	Pacrg	
NM_001099331	R3hdml	
NM_178045	Rassf4	CCCAGCTGCC:-1422;
NM_009162	Scg5	
NM_011352	Sema7a	ACCAGCTGGC:-658;GCCATCTGGC:-2433;TCCATCTGTT:-2302;
NM_019982	Sez6l	ACCAGATGGA:-177;GACAGATGGA:212;AACAGATGGG:-18;GACAGCTGGA:11;
NM_026886	Srrm4	ACCAGATGTC:-4828;
NM_009217	Sstr2	GCCATCTGCC:337;
NM_178874	Tmcc2	
NM_053267	Selm	ACCAGATGGC:1158;GGCATCTGTC:-4211;
NM_134050	Rab15	

Atoh1 E-Box Motif (AtEAM)

Figure 3: List of 23 candidate Atoh1 target genes obtained by cross-relational comparison of hair cell RNA-seq data and cerebellum Atoh1 ChIP-seq data. The AtEAM consensus binding site for Atoh1 is shown on the right; 12 of the candidate genes have AtEAM sites within 5kb of their coding regions. It is likely that some of the 11 other genes may have AtEAM sites located further away from their coding regions.

Several of these genes are known be to be expressed in hair cells (Barhl1, Hes6, Mfng, Selm, Rab15 and Atoh1 itself), but we are currently validating the expression of other genes in this list now.

Conclusions: *The Year 2-3 deliverable has been completed, although with a reduced number of Atoh1 targets, some of which require further validation in Year 3.*

Aim 2: To activate Atoh1 in damaged cochlear organ cultures to promote hair cell regeneration

We proposed the following deliverables for Year 2 and 3. As noted in last year's annual report, we made minor modifications to these deliverables based on our results in year 1:

1. We will determine whether activation of Atoh1 or Atoh1 and Gfi1 in cochlear supporting cells is able to drive them to a hair cell fate.
2. We will demonstrate whether hair cells generated by DAPT treatment and Atoh1 activation (or Atoh1 and Gfi1 activation) resemble *bona fide* hair cells by statistical comparison of these groups with wild type hair cells.

Results:

1. We are using the Cre-Lox transgenic mouse system to activate genes in mouse cochlear supporting cells. This requires mating a mouse in which a tamoxifen-inducible form of Cre recombinase is expressed in supporting cells to a second mouse in which the gene or genes to be activated are brought under the control of a Cre-inducible genetic locus.

As described in last year's annual report, we generated and screened several Cre lines, but did not identify a suitable line to activate gene expression strongly in supporting cells. This year, we obtained a new line of Sox2-CreEr mice from the Hochedlinger lab in Boston. These mice are now breeding well in our facility and in Figure 4, we show that these mice are able to drive strong gene expression in supporting cells in the presence of tamoxifen, which activates the CreER fusion protein.

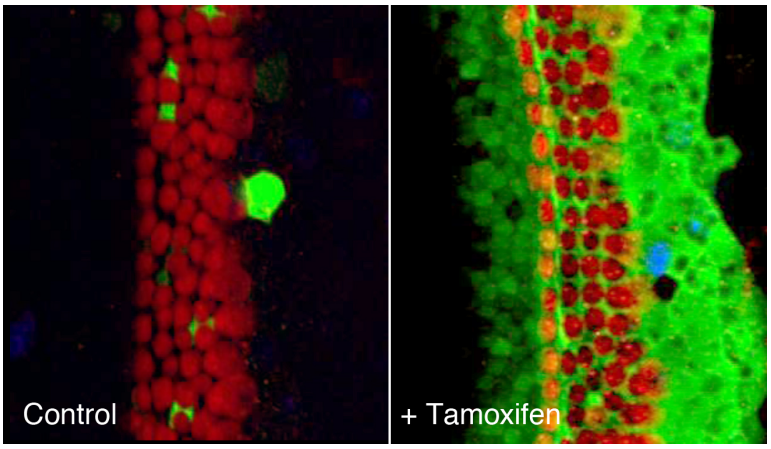

Figure 4: The new line of Sox2-CreER mice from the Hochedlinger lab give strong, specific recombination in supporting cells. The images are taken from cultured neonatal cochleas carrying the Sox2-CreER allele and a Cre reporter transgene which expresses green fluorescent protein in the presence of active Cre protein. Addition of tamoxifen to the cultures activates the Cre protein in supporting cells, giving strong GFP fluorescence. Hair cells are shown in red; the green fluorescence is in the supporting cell layer beneath the hair cells.

2. As described last year, we set out to make a line of transgenic mice in which Atoh1 could be expressed in a Cre-inducible fashion. At the start of this project year, we began screening ES cell lines that contained a Cre-inducible version of Atoh1 (Math1) inserted into the ROSA locus (Figure 5). However, as described in our last quarterly report, our initial attempts to target the ROSA locus with this construct were unsuccessful. This was due to a technical problem with identifying successful homologous recombination at the ROSA locus with Southern blotting. We have now re-designed this screening strategy, and have now successfully completed the targeting in ES cells. These mice should be ready for screening in the next 6 months.

In our last quarterly report, we stated that two groups have recently published papers showing that the ability of Atoh1 to induce new hair cells drops dramatically after the onset of hearing. This suggests that other factors may be required to co-operate with Atoh1 to drive hair cell differentiation in the mature ear. In collaboration with the Raphael group at the University of Michigan, we have evidence that co-expression of the Atoh1 and Gfi1 transcription factors can generate more hair cells and for longer periods than Atoh1 alone. In parallel with the Atoh1-expressing mice described above, we have also engineered a targeting construct to express *both* Atoh1 and Gfi1 and equal levels in a Cre-inducible fashion. We feel it

will be important to directly compare the effects of expressing Gfi1 and Atoh1 together with that of expressing Atoh1 alone, as it will allow a direct comparison of the efficiency of both approaches.

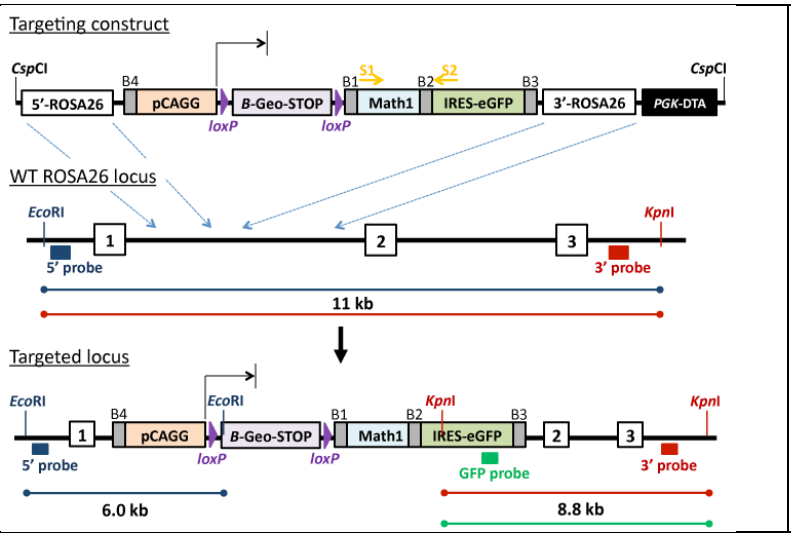

Figure 5: Diagram showing the targeting construct (top) designed to target the mouse ROSA locus (middle) by homologous recombination in ES cells. This should lead to a correctly targeted, Cre-inducible form of Atoh1 (bottom) in which GFP will also be expressed from an internal ribosome entry site (IRES). This diagram was also provided in the previous Annual Report; we provide it here again for clarification.

The targeting construct used to express both Atoh1 *and* Gfi1 is exactly the same as that shown in Figure 5, except that the Math1/Atoh1 gene has been replaced with a sequence containing Atoh1, followed by a picornavirus 2A sequence to allow co-translation and then the Gfi1 sequence. Using the modified screening approach described above, we successfully targeted ES cells with this construct and have now generated 14 chimeric mice which we are waiting to screen for germline transmission. *We will know if these mice are working within the next 12 weeks.*

3. Our approach to regenerate hair cells by expressing Atoh1 in supporting cells requires that the newly formed hair cells survive. In recent work performed in the last quarter, we have shown that newly-formed hair cells have an *absolute requirement* for Atoh1 shortly after they differentiate. We have shown that there is a critical time period of about 3 days after hair cells form, in which they will rapidly die if Atoh1 is removed by gene deletion. *This suggests that methods to replace hair cells by activating Atoh1 must be robust enough to ensure expression of Atoh1 for longer than this "critical period".*

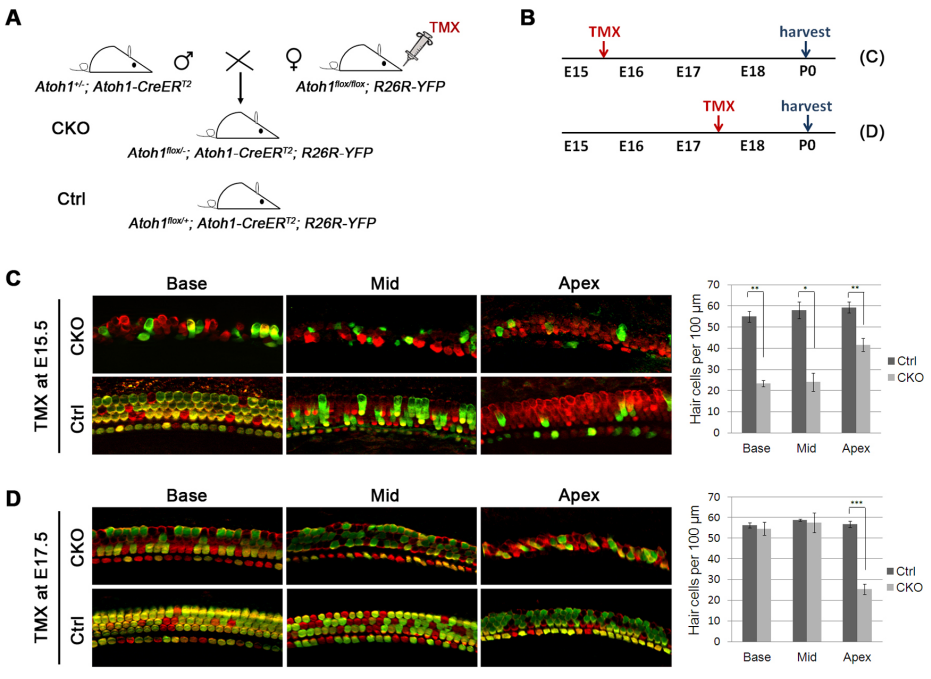

Figure 6: There is a critical period for Atoh1 in hair cell survival after differentiation. A: The mating strategy used to delete Atoh1 in hair cells at different points after they differentiate. Briefly, and injection of tamoxifen into pregnant mothers causes the Atoh1 gene to be deleted and a GFP reporter to be expressed in hair cells. B: We deleted Atoh1 at E15.5 and E17.5, two and four days after hair cells in the basal region of the cochlea differentiate. C and D: Deletion of Atoh1 at E15.5 causes significant hair cell death along the cochlea. However, by E17.5, the oldest hair cells in the base- and mid-turns of the cochlea survive after Atoh1 deletion, suggesting there is an approximately 3 day critical period for Atoh1 after new hair cells are formed.

4. As discussed in our Year 2 quarterly reports, we showed that although blocking the Notch signaling pathway can cause supporting cells to transdifferentiate into hair cells, this effect is only seen in neonatal mice, with virtually no response occurring in one week old mice (Figure 7).

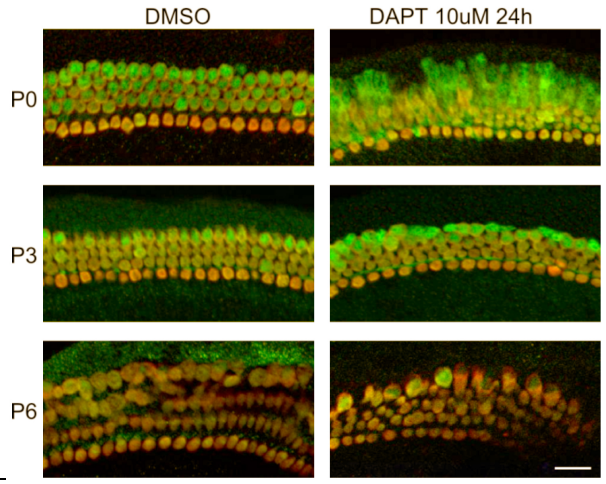

Figure 7: The Notch pathway blocker DAPT can convert supporting cells into hair cells, but this effect declines rapidly with age. Cochlear organ cultures were established from newborn (P0) , 3 day old (P3) and 6 day old (P6) mice and cultured for 24 hours in DAPT or DMSO as a vehicle control. Hair cells are shown with Myosin7a (red) and Atoh1-GFP (green). Significant numbers of new hair cells are seen in P0 cultures, but very few in older cultures.

We proposed to analyze the transcriptional changes in supporting cells from neonatal and one week old animals to see which genes are affected by DAPT treatment, and whether other sets of genes are activated at later stages when hair cell differentiation cannot be induced. To do this, we are using Lunatic Fringe-GFP transgenic mice, which express GFP in a restricted population of supporting cells in both newborn and one week old cochleas. These mice can be used to purify supporting cells from the cochleas of both ages of mice (Figure 8):

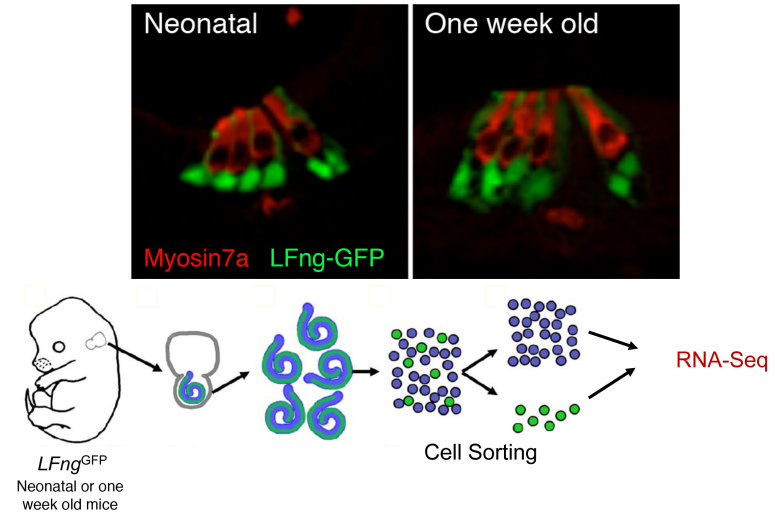

Figure 8: The Lunatic Fringe (LFng)-GFP transgenic mice express GFP in supporting cells, but not hair cells, from newborn and one week old mice. The transgene labels all Deiters' cells, outer pillar cells, inner phalangeal cells and border cells. To purify supporting cells from these mice, we isolate cochlear epithelium, dissociate the cells into single cells and isolate the GFP+ cells by fluorescent activated cell sorting (see previous Annual Report).

We have now made RNA sequencing libraries from purified supporting cells from both newborn and 6 day old cochleas and the sequencing of these libraries is now being completed and analyzed by Baylor's Human Genome Sequencing Center. Analysis of these libraries will be completed by the end of November.

We have also exposed newborn and six day old cochleas to pharmacological blockers of Notch signaling and have purified supporting cells after 24 hours of Notch blockade. This will allow us to determine the transcriptional changes that occur (or fail to occur) at the two different ages. We have collected all the cells and RNA we need from these samples, and library construction and sequencing will commence after our first set of samples have been analyzed.

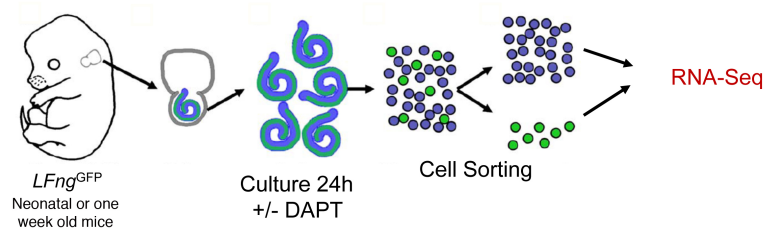

Figure 9: To identify genes up- or down-regulated in supporting cells after exposure to the Notch blocker DAPT, cochleas from LFng-GFP mice are cultured for 24 hours in the presence of DAPT or DMSO control, and the cochleas are then dissociated and the supporting cells purified as shown above.

Conclusions: *We are halfway through completion of the Year 2-3 deliverables. The delay in generating appropriately targeted transgenic mice has set back progress on these aims. We propose to complete the RNA-seq experiments in year 3, but we are likely to request an extension of the project into a fourth year to complete the transgenic mouse work. This will be determined in conjunction with our Grants Officer's Representative*

Aim 3: To activate Atoh1 in deafened mice to promote hair cell regeneration

We proposed the following deliverable for Year 2:

Year 2 deliverables: We will verify that the Sox2-CreER/dnMAML system is able to generate new hair cells in our hands.

1. Mastermind (or MAML) is an essential co-factor in the Notch signaling pathway. It is absolutely required for Notch signaling, and loss of MAML causes phenotypes very similar to loss of Notch function. There are three members of the MAML family in vertebrates, and as a result, strategies to disrupt MAML function have used truncated forms of the MAML1 gene that act as dominant mutations and therefore block all MAML activity. Our goal is to test the effects of blocking Notch activity in the cochlea by activating expression of this dominant MAML1 mutant (dnMAML) in supporting cells with Sox2-CreER mice. Our prediction is that this may turn supporting cells into hair cells.

As mentioned above, we have only recently confirmed that the second line of Sox2-CreEr mice from the Hochedlinger lab in Boston is working well to drive gene expression in cochlear supporting cells. In the meantime, we have begun testing the dnMAML1 transgenic mice in the lab by activating its expression throughout the cochlea using UBC-CreER transgenic mice. As described in the last quarterly report, our initial experiments with these mice generated heterozygous pups that had one copy of the dnMAML allele. These mice did not have significantly greater numbers of hair cells than their wild type littermates. In the last quarter, we have tested the possibility that gene dosage may be responsible for this result by generating homozygous pups carrying two copies of the dnMAML allele**.**

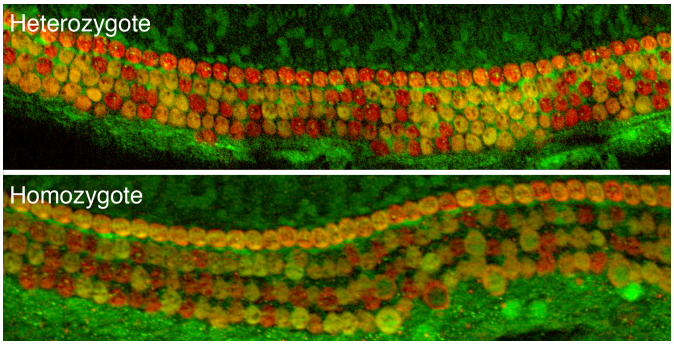

Figure 10: Neither heterozygous nor homozygous dnMAML mice generate significant numbers of ectopic hair cells. Images show cultured newborn cochleas from a cross of UBC-CreER; dnMAML1$^{flox/+}$ x dnMAML1 $^{flox/flox}$ mice. Mice were genotyped to determine which pups were homozygous and heterozygous. Cochleas were treated with tamoxifen to activate dn MAML1 expression. Red fluorescence is Myosin7a, while activation of the dnMAML1 allele is shown with green fluorescence. Although occasional extra outer hair cells can be observed, *we see no significant differences between the two genotypes.*

We were surprised at these results, as in neither genotype gave significant numbers of ectopic hair cells. This contrasts with our experiments with newborn cochleas treated with the Notch inhibitor DAPT (Figure 7) which show an approximately 50% increase in hair cells.

We have proposed two explanations for this result. The first is that the dnMAML1 mutant mice are not effectively inhibiting Notch signaling in the cochlea, perhaps because the levels of the mutant MAML1 protein are not expressed at high enough levels. However, these same mice have been sued to effectively inhibit Notch signaling in a variety of other tissues during development and in the adult animal. An alternative explanation is that the ectopic hair cell production we observe with DAPT is not due to the inhibition of the Notch pathway. DAPT is an inhibitor of the gamma secretase complex, an enzyme complex that is important for cleavage of the Notch receptor, but also for other transmembrane receptors such as the amyloid precursor protein APP. It is therefore conceivable that the hair cell phenotype is due to inhibition of another membrane protein that can regulate hair cell development.

We are currently testing this in several ways. First, we are examining the expression of Notch pathway genes in our dnMAL1 mice and in cochleas that have been treated with DAPT. Second, we are attempting to reproduce our DAPT experiments with new blocking antibodies to the Notch receptor that we have obtained in an agreement with the pharmaceutical company Genentech. Third, we are attempting to inhibit Notch signaling at embryonic times with the dnMAML1 mice to see if there is an age-dependent decline in its potency in the cochlea.

The outcome of these experiments is critical, as they will determine whether we proceed with attempts to promote hair cell regeneration through the Notch pathway or through another signaling pathway that is also regulated by the gamma secretase complex.

Conclusions: *We have not yet completed this deliverable due to a delay in the Sox2-CreER mice and the possibility that the hair cell phenotype we are seeing maybe unrelated to Notch activation. We propose to complete these experiments in year 3, but we may require an extension of the timeline into a fourth year to complete experiments. This will be determined in conjunction with our Grants Officer's Representative*

KEY RESEARCH ACCOMPLISHMENTS:

• Developed techniques to purify supporting cells from cultured organ of Corti

• Generated a validated list of genes expressed in hair cells using RNA-seq

• Generated a high-quality RNA-Seq library from neonatal and six day old supporting cells.

• Completed material collection to compare the effects of DAPT on neonatal and six day old supporting cells

• Validated RNA-Seq library construction on small numbers of cells

• Confirmed that the new line of Sox2-CreER mice is able to efficiently cause gene activation or deletion in cochlear supporting cells.

• Generated a targeting construct to conditionally activate Atoh1 and Gfi1 in any cell or tissue type in the mouse. Established 14 founder mice that are being checked now for germline transmission

REPORTABLE OUTCOMES:

Informatics: We have compiled two databases of genes whose expression is enriched in hair cells by both microarray and RNA-seq. We have cross-referenced these databases to extract genes in these lists that contain Atoh1-binding sites within 5kb upstream or downstream by bioinformatic interrogation with a consensus AtEAM site. We have established that the two different methods give distinct but overlapping results

ES cell production: We have generated successfully targeted ES cell lines to generate Cre-inducible forms of Atoh1 and both Atoh1 and Gfi1 simultaneously. We have also generated adenoviral expression constructs to express these genes in the organ of Corti if required.

Transgenic mouse production: We have generated 14 founder mice to activate Gfi1 and Atoh1 in supporting cells. We will be testing for germline transmission of these founders in the next 6 weeks as they reach reproductive age.

CONCLUSION:

The long-term goal of this project is to use activation of the Atoh1 gene by pharmacological or genetic means to promote hair cell production in the damaged cochlea as a means of hearing restoration.

The three goals of the proposal are to identify the genetic targets of Atoh1 and to demonstrate as a proof of principle that activation of Atoh1 can generate hair cells in organ culture and transgenic mouse models. Much of our efforts in Year 2 were devoted to collecting material for RNA-Seq libraries from neonatal and six day old supporting cells, and from supporting cells that had been treated with DAPT in culture for 24 hours. These experiments will allow us to identify genes that change in supporting cells with age and after blockade of the Notch pathway.

The second goal for Year 2 was to complete the generation of transgenic mice for our in vivo studies. Although progress has been made with the generation of targeted founder mice and the establishment of the Sox2-CreER mouse, some technical issues means that we will likely require an extension of the proposal period to complete these experiments in a 4th year.

Finally, we are exploring the possibility that was raised throughout year 2's work that inhibition of the Notch signaling pathway may not promote hair cell generation from supporting cells, but rather, that another gamma secretase-dependent pathway is responsible for the effects of DAPT. This has the potential to reveal a novel pathway for hair cell regeneration, and so we are very interested in pursuing this avenue of research.